




This is the **accepted version** of the article:

Wang, Ruzhen; Cao, Yanzhuo; Wang, Hongyi; [et al.]. «Exogenous P compounds differentially interacted with N availability to regulate enzymatic activities in a meadow steppe». European Journal of Soil Science, Vol. 71, issue 4 (July 2020), p. 667-680. DOI 10.1111/ejss.12906

This version is available at <https://ddd.uab.cat/record/232204>

under the terms of the  **IN COPYRIGHT** license

Exogenous P compounds differentially interacted with N availability to regulate enzymatic activities in a meadow steppe

Ruzhen Wang^{a,1,*}, Yanzhuo Cao^{a,b,1}, Hongyi Wang^{a,c}, Feike A. Dijkstra^d, Jinglun Jiang^a, Ruonan Zhao^a, Wang Ma^a, Tianpeng Li^a, Maxim Dorodnikov^e, Zhengwen Wang^a, Jordi Sardans^{f,g}, Josep Peñuelas^{f,g}

^a*Erguna Forest-Steppe Ecotone Ecosystem Research Station, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, China*

^b*Key Laboratory of Regional Environment and Eco-Remediation, College of Environment, Shenyang University, Shenyang 110044, China*

^c*Heilongjiang Bayi Agricultural University, Daqing 163319, China*

^d*Sydney Institute of Agriculture, School of Life and Environmental Sciences, The University of Sydney, Sydney, NSW 2006, Australia*

^e*Department of Soil Science of Temperate Ecosystems, Georg-August University of Göttingen, Büsgenweg 2, 37077 Göttingen, Germany*

^f*CSIC, Global Ecology Unit CREAF-CSIC-UAB, 08913 Bellaterra, Catalonia, Spain*

^g*CREAF, 08913 Cerdanyola del Vallès, Catalonia, Spain*

¹*Authors contributed equally to this work.*

*Corresponding author: Ruzhen Wang, Tel: +86 24 83970603; Fax: +86 24 83970300

E-mail address: ruzhenwang@iae.ac.cn

Summary

Increased inputs of ecosystem nitrogen (N) and phosphorus (P) may affect the activity of soil enzymes that play essential roles in the metabolization of carbon (C), N and P for microbial growth. However, the associations between soil enzymatic activities and N and P availability remain poorly understood. We conducted a study in a meadow steppe to elucidate the effects of the addition of N, as ammonium nitrate (NH_4NO_3), and two forms of P with contrasting solubility, comprising monopotassium phosphate (KH_2PO_4) that is more soluble than triple superphosphate ($\text{Ca}(\text{H}_2\text{PO}_4)_2$), on activity of β -glucosidase (BG), *N*-acetyl-glucosaminidase (NAG) and acid and alkaline phosphomonoesterases (PMEs). In general, there was a positive effect of N on BG, NAG and alkaline PME activity as a result of enhanced soil N availability, plant-microbe nutrient competition and plant P uptake. Addition of KH_2PO_4 increased activity of BG, NAG and alkaline PME, but had no impact on acid PME activity. Addition of $\text{Ca}(\text{H}_2\text{PO}_4)_2$ increased NAG activity, but only increased activity of BG and alkaline PME with the addition of N. Concentration of soil available P and microbial biomass P increased with added P, particularly KH_2PO_4 . These results provide the first evidence for the N- and P-mediated stimulation of microbial activity depending on the chemical form of added P in this ecosystem. Relationships between activity of BG and NAG, and between that of NAG and PME were allometric, indicating disproportionate changes in activity of these soil enzymes. This further suggests shifts in microbial acquisition of C, N and P along with increases in availability of N and P that may potentially affect plant productivity. We conclude that scenarios of global environmental change, in which

ecosystem availability of N and P are affected, may result in variable activity responses among soil enzymes, while the chemical form of P input should be considered as an important factor influencing meadow steppe grassland ecosystem function.

Keywords enzymatic stoichiometry, extracellular enzymatic activity, microbial biomass phosphorus, nitrogen availability, phosphorus fertilization

Highlights

- Chemical N and P increased enzyme activity but effects varied with P form and rate.
- N addition promoted soil enzyme activity through enhanced plant-microbe interactions.
- P and N availability resulted in variable activity responses among soil enzymes.
- Enzymatic stoichiometry showed varying microbial responses in C-, N- and P-acquisition.

Introduction

Current inputs of nitrogen (N) to ecosystems are 2- to 3-fold greater than levels prior to the green revolution and are >4-fold greater than those of phosphorus (P) (Peñuelas *et al.*, 2013; Wang *et al.*, 2018). Although global anthropogenic N inputs have steadily increased from 120-150 Mt y⁻¹ in the 1980s to 165-250 Mt y⁻¹ in the 2000s (Peñuelas *et al.*, 2012) and are largely derived from crops that fix N₂, industrial fertilizers and emissions from fossil fuels, global anthropogenic inputs of P, which mostly stem from fertilizer use, have remained relatively stable. Thus, anthropogenic inputs of N and P have become increasingly unbalanced, with N:P ratios that are often much greater than those for terrestrial plants (Peñuelas *et al.*, 2012, 2013). Changes in N and P cycles influence ecosystem stability and functions, such as primary productivity, plant-litter decomposition, nutrient release and C balance, particularly in temperate and boreal (limited by N) and tropical (limited by P) regions (Peñuelas *et al.*, 2013; Fernández-Martínez *et al.*, 2014; Jing *et al.*, 2016; Niu *et al.*, 2016; Chen *et al.*, 2017).

Soil enzymes play a key role in the decomposition of soil organic matter and recycling of soil nutrients for plant and microbial growth (Shukla & Varma 2011; Trivedi *et al.*, 2016). For example, β -glucosidase (BG) enzymes, which hydrolyze cellulose and other β -linked glucans into glucose, and N-acetyl-glucosaminidase (NAG) enzymes, which hydrolyze chitin and other β -linked aminopolysaccharides into glucosamine, are commonly used indicators of microbial C and N acquisition, respectively (Carreiro *et al.*, 2000; Sinsabaugh *et al.*, 2014). Acid and alkaline phosphomonoesterases (PMEs), required to hydrolyze phosphate from phospholipids

and phosphosaccharides are used as indicators of microbial P acquisition (Sinsabaugh *et al.*, 2014; Jian *et al.*, 2016). β -glucosidase~~BG~~, NAG and acid/alkaline PME catalyze terminal and rate-limiting reactions to produce C, N and P products that are assimilable by microbes, so their activities represent microbial C, N and P demand (Tabatabai, 1994; Sinsabaugh *et al.*, 2014). As a result, these four enzymes have been used in studies to improve understanding of C, N and P cycling in soils (Sinsabaugh *et al.*, 2009, 2014; Waring *et al.*, 2014; Cenini *et al.*, 2015). Soil C-cycling enzymes regulate the activity of N- and P-cycling enzymes via influencing microbial C availability and consequentially enzymatic activity, so activities of these enzymes are often tightly coupled with stoichiometric relationships (Waring *et al.*, 2014). Activities of soil enzymes have been shown to be positively correlated with plant productivity (Margalef *et al.*, 2017; Sterkenburg *et al.*, 2018), plant nutrient demand (Sardans *et al.*, 2007) and soil C:N:P stoichiometry of an ecosystem (Sinsabaugh *et al.*, 2009), and may be affected by anthropogenic mediated changes in ecosystem availability of N and P. However, the magnitude and direction of single and combined effects of N and P inputs remain uncertain.

Effects of N addition on soil enzymatic activities in grassland ecosystems have been widely studied (summarized in Wang *et al.* 2014~~), and~~ and have been shown to vary. For example, positive, negative and neutral effects of soil N availability on C- and P-cycling enzyme activities have been reported (refer to Wang *et al.*, 2014), indicating that enzyme activity may be mediated by other soil physicochemical properties, such as soil pH, moisture and P availability (Sardans *et al.*, 2007; Sinsabaugh *et al.*, 2008;

Waring *et al.*, 2014; Mori *et al.*, 2018). Indeed, N-induced soil acidification has been found to decrease BG and PME activities by inhibiting microbial growth, but higher levels of N availability led to reduced limitation of microbial N (Yang *et al.*, 2017). Effects of P addition, particularly those related to chemical form of P, on enzymatic activities (Table 1) and potential ecosystem responses in grasslands are less clear than those of N. For example, the addition of the relatively soluble monosodium phosphate (NaH_2PO_4) increased plant productivity on average by 22% in 98 grassland soils across North America (Craine & Jackson, 2010), while the less soluble triple superphosphate ($\text{Ca}(\text{H}_2\text{PO}_4)_2$) elicited positive (+59%) and neutral effects in South African grasslands (Craine *et al.*, 2008). ~~and~~ And additions of NaH_2PO_4 and $\text{Ca}(\text{H}_2\text{PO}_4)_2$ decreased (Phoenix *et al.*, 2003) and increased (Tian *et al.*, 2016) PME activity in natural calcareous grasslands, respectively.

These contrasting effects of different chemical forms of P on enzyme activity may be due to differences in soil pH, P-absorption capacity, time since application and ~~their~~ varying use efficiencies by plants and soil microorganisms related to P compound chemistry (Mori *et al.*, 2018). For example, KH_2PO_4 addition was found to increase BG, NAG and acid PME activities in both a savannah and a semi-natural grassland (Mganga *et al.*, 2015), while superphosphate application had no impact on the activity of the three enzymes in an alpine grassland (Jing *et al.*, 2016). However, no comparison has been made for the differential effects of soluble and less soluble P forms on enzyme activities in the same grassland ecosystem with the same environmental conditions and fertilization history. Pre-input levels of ecosystem N and P are essential factors that

influence microbial activity in response to P inputs (Waring *et al.*, 2014; Tian *et al.*, 2016; Margalef *et al.*, 2017), as demonstrated by increased PME activity in a P-poor steppe in response to P addition, but decreased activity in a relatively P-rich old-field grassland (Tian *et al.*, 2016). Although little is known about the combined effects of inputs of N and chemical forms of P on C-, N- and P-cycling enzyme activities, microbial economic theory suggests that higher levels of P availability suppress P-cycling enzyme activity, but promote C- and N-cycling enzyme activities (Allison *et al.*, 2010), where these effects would be stronger with more soluble forms of P.

The meadow steppe of northeastern China represents one of the dominant types of grassland in Eurasia and plays a vital role in supporting the regional economy, floral diversity and environmental health (Wang & Ba, 2008). This cold meadow steppe is highly sensitive to global climate change and is within the vulnerable ecotone between forest and steppe that receives 1-2 g N m⁻² y⁻¹ (Xu *et al.*, 2015). Given increased productivity of the grassland is required to support a growing human population, more efficient P fertilization is urgently needed to prevent the gradual depletion of natural P reserves (Sattari *et al.*, 2016). Therefore, we investigated the effects of combined additions of N and chemical forms of P on ecosystem processes in a meadow steppe field experiment, to test the hypotheses that (1) combined P and N addition ~~would~~ alleviate the decrease of NAG and increase of PME activities but stimulate the increase of BG activity as caused by N addition alone due to increasing microbial C and N requirements with enhanced P inputs; and (2) Ca(H₂PO₄)₂ ~~would have~~ less impact on enzyme activities than KH₂PO₄ where increases in BG and NAG activities and

decreases in PME activities are more pronounced under the more soluble form of P.

Materials and methods

Study site and experimental design

The field experiment was conducted at the Erguna Forest-Steppe Ecotone Research Station, Chinese Academy of Sciences, located at the southern boundary of the Eurasian permafrost region in Inner Mongolia. The climate of this area belongs to the transition zone between cold- and mid-temperate climates, with mean annual temperature and precipitation of -3 °C and 375 mm, respectively. The meadow steppe ecosystem is dominated by *Leymus chinensis* (Trin.) Tzvel, *Stipa baicalensis* Roshev and *Carex duriuscula* C.A.Mey, and soils are classified as Chernozem (IUSS Working Group WRB 2014). The relatively low background inputs of N and P render this an ideal location for the study of ecosystem responses to global environmental change and nutrient management. The bulk soil pH was 6.8 ± 0.07 . Elemental analysis showed the bulk soil to have 24.0 ± 0.57 g kg⁻¹ C, 1.8 ± 0.06 g kg⁻¹ N and 0.5 ± 0.02 g kg⁻¹ P.

The experiment started in May 2014, and annual applications of fertilizers in May comprised KH₂PO₄ or Ca(H₂PO₄)₂ applied at 0, 2, 4, 6, 8 and 10 g P m⁻² y⁻¹, with (elevated) and without (ambient) N, applied as NH₄NO₃ at 0 and 10 g N m⁻² y⁻¹, arranged as three factors in a randomized block design, with five replicates; blocks were separated by 2-m buffer strips, and the 24 plots (8 × 8 m) within a block were separated by 1-m buffer strips (Figure S1). Fertilizers were applied to the soil surface as pellets. We chose 10 g N m⁻² y⁻¹ as it has been suggested to be the upper threshold for affecting

aboveground productivity, species richness and composition of plant functional groups in Inner Mongolian grasslands (Bai *et al.*, 2010). However, an upper threshold for P has not been clearly established. In this study, we chose two ~~commonly used~~commonly used P fertilizers of KH_2PO_4 and $\text{Ca}(\text{H}_2\text{PO}_4)_2$ to compare their effects on soil enzyme activities. We used KCl in the KH_2PO_4 plots to ensure similar levels of potassium (K) that were equal to those in the highest P treatment (13.2 g K m^{-2}); CaCl_2 was used to maintain a constant annual chloride (Cl) input (12.1 g Cl m^{-2}) with KCl addition. Calcium was not compensated, because of its high natural abundance in the calcareous soils.

Field sampling

Aboveground biomass of all plant material, including litterfall, was harvested from a $1 \times 1\text{-m}$ quadrat that was placed randomly in each plot in August 2016. Plants were sorted to species and oven-dried with the litterfall biomass at 65°C for 48 h, and then weighed. Five samples of soil, which were collected from the 0-10 cm layer using a 5-cm diameter auger in August 2016, were combined to form a single composite sample per plot and then stored in insulated cans at 4°C during transport to the laboratory. The soil samples were sieved through a 2-mm screen to remove stones and visible roots; samples were subdivided, where one subsample was stored at 4°C until analysis of microbial parameters, and another was air-dried.

Soil pH was determined in a 1:5 (w/v) soil-water suspension using a PHS-3G digital pH meter (Precision and Scientific Instrument Co. Ltd., Shanghai, China). A subsample

of the air-dried soil was ground using a ball mill (Retsch M400, [Haan](#), Germany) for analysis of soil organic C (SOC), total N (TN) and total P (TP). SOC and TN concentrations were determined using $K_2Cr_2O_7$ oxidation (Nelson & Sommers, 1982) and the Kjeldahl method (Bremner, 1996), respectively, and soil TP concentration was determined using molybdenum-blue colorimetry following acid digestion of 0.1 g of soil (HNO_3 , $HClO_4$ and HF). [Total](#) P concentration in the three dominant plant species (*L. chinensis*, *S. Baicalensis* and *C. duriuscula*) was determined using molybdenum-blue colorimetry following acid digestion of 0.3 g of plant material (HNO_3 and $HClO_4$) (Murphy & Riley, 1962) and P uptake in the species was determined as the product of its biomass and P concentration. Total inorganic N (TIN) concentration was calculated as the sum of NO_3^- -N and NH_4^+ -N concentrations that were determined using a continuous-flow analyzer (AutoAnalyzer III, Bran & Luebbe, Norderstedt, Germany), following extraction from fresh soil using 2 M KCl.

Microbial biomass C (MBC) was determined using fumigation-extraction (Vance *et al.*, 1987), where a 15-g subsample of fresh soil was fumigated with chloroform for 24 h; another 15-g subsample was not fumigated. Following extraction with 0.5 M K_2SO_4 , the concentration of C was determined using a TOC analyzer (Multi N/C 3100, Analytik Jena, Jena, Germany), and MBC concentration was calculated as the difference between the fumigated and unfumigated samples. To correct for incomplete extraction, an efficiency factor of 0.38, which has previously been used for this grassland, was used to calculate actual MBC concentration (Wang *et al.*, 2015). Concentration of microbial biomass P (MBP) was determined as for MBC, except we used 0.5 M $NaHCO_3$ as the

extractant (Brookes *et al.*, 1982). Phosphate concentration in the extracts was measured using molybdenum-blue colorimetry with a UV-visible spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) (Murphy & Riley, 1962). The efficiency factor for MBP (0.39) was determined according to the equation proposed by Bilyera *et al.* (2018), using SOC (24.5 g kg⁻¹), total P (0.53 g kg⁻¹) and clay content (23.6%). Extractable C in the unfumigated samples was classed as dissolved organic C (DOC), and available (Olsen) P was extracted from 2.5 g of air-dried soil with 50 ml of 0.5 M NaHCO₃ (pH 8.5) (Olsen *et al.*, 1954) and then filtered; Olsen P was measured using molybdenum-blue colorimetry. Biomass of arbuscular mycorrhizal fungi (AMF) was estimated from analysis of phospholipid fatty acids that were extracted, fractionated and quantified (after Bossio & Scow, 1998) from frozen soil samples; the extraction mixture comprised CHCl₃, methanol and citrate buffer (1:2:0.6). The extracted phospholipids were separated from neutral lipids and glycolipids and then methylated into fatty acid methyl esters via a mild alkaline methanolysis; the fatty acid methyl esters were then analyzed using a gas chromatograph (Agilent 7890A, Wilmington, USA) and identified using a microbial identification system (Microbial ID. Inc., Newark, DE, USA). We used fatty acid 16:1 ω 5 as a biomarker for AMF (Zhang *et al.*, 2015).

Soil enzymatic activity

Maximum potential activities of BG, NAG and acid/alkaline PME required to catalyze specific reactions were determined at their respective optimal pHs and temperatures from fresh soil samples to allow comparison with other studies. The incubation

temperature was not adjusted to the mean annual temperature (-3 °C) that may have been a rate-limiting factor and obscured any treatment effects (Nannipieri *et al.*, 2018). For BG activity, 1 g of soil was mixed with 0.25 ml of toluene, 4 ml of modified universal buffer (comprising 0.1 M tris(hydroxymethyl)aminomethane, 0.067 M citric acid monohydrate and 0.1 M boric acid; pH 6.0) and 1 ml of 0.5 M *p*-nitrophenyl- β -D-glucopyranoside (CAS:2492-87-7) substrate; the mixture was incubated at 37 °C for 1 h, and the reaction was stopped by adding 1 ml of 0.5 M CaCl₂ and 4 ml of 0.1 M tris(hydroxymethyl)aminomethane (pH 12.0). Then, the mixture was filtered and production of *p*-nitrophenol (PNP) was measured colorimetrically using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) at 410 nm. Activities of NAG and acid/alkaline PME were measured as for BG activity, except we used a different substrate and pH for the reaction system: we used *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminidine, *p*-nitrophenyl phosphate and *p*-nitrophenyl phosphate as substrates buffered at pHs of 5.5 (Parham & Deng, 2000), 6.5 and 11.0 (Tabatabai *et al.*, 1994), respectively. Activities were expressed as the rate of PNP production (mg PNP kg soil⁻¹ h⁻¹). We acknowledge that our enzyme “activity” measurements only provide an indication of enzyme concentrations and do not represent actual soil enzyme activities (Wallenstein and Weintraub 2008).

Statistical analyses

Data were tested for normality using the Kolmogorov-Smirnov test, and homogeneity of variance was determined using Levene’s test; data were log-transformed for analysis

of variance (ANOVA) as appropriate. Three-way ANOVA, with N addition, rate of P addition and chemical form of P fertilizer as factors, was used to test for treatment differences in soil physicochemical properties and enzymatic activities. Associations between enzymatic activities and physicochemical properties were tested using Pearson correlation analysis. Statistical analyses were performed in SPSS 19.0 for Windows (SPSS Inc., Chicago, USA). Redundancy analysis (RDA) (Canoco for Windows 5.0, Plant Research International, Wageningen, The Netherlands) was used to determine the relationships between soil physicochemical properties and enzymatic activities; prior to analysis, enzymatic activity data were $\log_{10}(x+1)$ -transformed to correct for positive skewing, and the soil physicochemical properties were zero-centered for data standardization. We used a Monte Carlo test (499 permutations) (Canoco for Windows 5.0, Plant Research International, Wageningen, The Netherlands) to determine the relative contribution of soil parameters to variation in enzymatic activities, and the relationship between BG, NAG and acid PME enzymatic activities was tested using standardized major-axis (SMA) regression in R software (<http://www.r-project.org>, last accessed: February 2018) and compared with regression slopes of unity to identify isometric (not different from unity) or allometric (different from unity) relationships at $P < 0.05$.

Results

Effects of N and P on soil physicochemical properties

Soil pH decreased with addition of $\text{Ca}(\text{H}_2\text{PO}_4)_2$ under ambient and elevated levels of N

input ($P < 0.01$; Tables S1 and S2) due to release of H^+ during hydrolysis of $Ca(H_2PO_4)_2$. There was a tendency towards a decrease in SOC concentration with increasing rate of KH_2PO_4 , irrespective of N treatment ($P = 0.06$). Addition of N increased TIN concentration, regardless of form of P ($P < 0.01$; Table S2). TP concentration was greater with increasing rate of KH_2PO_4 , irrespective of addition of N, and with addition of $Ca(H_2PO_4)_2$, without N (Tables S1 and S2). Olsen-P concentration was greater with increasing rate of P, irrespective of form of P and addition of N ($P < 0.01$; Table S2). We found that MBP concentration was greater with increasing rate of added P, regardless of form of P and addition of N ($P < 0.01$; Figures 1a and b, Table S1) and it was greater under KH_2PO_4 than $Ca(H_2PO_4)_2$ ($P < 0.01$; Table S3, Figure 1). In general, there was a negative effect of N on AMF biomass (Figure S2a).

Effects of N and P on enzymatic activities

In general, addition of N positively affected BG, NAG and alkaline PME activities, while there was an interaction between the effects of N and rate of P on acid PME activity (Figure 2, Table S3). ~~β -glucosidase~~BG activity increased with higher rates of KH_2PO_4 under ambient N ($P < 0.01$; Figure 2a) and at 4, 8 and 10 $g\ m^{-2}\ y^{-1}$ KH_2PO_4 and 6 $g\ m^{-2}\ y^{-1}$ $Ca(H_2PO_4)_2$ under elevated N (Figures 2a and b). The higher rates of P (6-10 $g\ m^{-2}\ y^{-1}$) increased NAG activity, regardless of N and form of P ($P < 0.01$; Figures 2c and d). Acid PME activity was not affected by the addition of KH_2PO_4 , irrespective of N (Fig. 2e), and increased with addition of $Ca(H_2PO_4)_2$ with elevated N (Figure 2f). Alkaline PME activity increased with increasing rate of KH_2PO_4 , irrespective of N

(Figure 2g) and with rate of $\text{Ca}(\text{H}_2\text{PO}_4)_2$ under elevated N ($P < 0.01$; Figure 2h). Chemical form of P affected alkaline PME activity ($P < 0.05$; Table S3), where average activity across rates of P and N was greater with added $\text{Ca}(\text{H}_2\text{PO}_4)_2$ than KH_2PO_4 . We found positive associations between BG and NAG ($P < 0.01$; Figure 3a, Table S4) and between NAG and acid PME ($P = 0.02$; Figure 3f, Table S4), and all slopes in the SMA regression differed from 1.0, indicating allometric relationships between enzymatic activities (Table S4).

Effects of N and P on plant and soil function

Aboveground plant biomass increased with elevated N and, when averaged across rates of P and N treatment, was greater with addition of KH_2PO_4 than $\text{Ca}(\text{H}_2\text{PO}_4)_2$ ($P = 0.036$; Figure S2b). Phosphorus uptake in the three plant species was greater with addition of KH_2PO_4 than $\text{Ca}(\text{H}_2\text{PO}_4)_2$ ($P = 0.02$; Figure S2c), with increasing rate of added P ($P < 0.01$) and with elevated N ($P < 0.01$). Addition of N and P resulted in greater levels of aboveground litterfall biomass (Figures 4a and b).

β -glucosidase activity was positively correlated with litterfall biomass, regardless of form of P and addition of N (Figures 4c and d), while under addition of KH_2PO_4 , it was positively correlated with TP (Figure S3a) and Olsen P (Figure S3b) concentrations and negatively associated with total C:P ($P = 0.01$; Figure S3c) and total N:P ($P = 0.01$; Figure S3d) ratios.

N-acetyl-glucosaminidase activity was positively correlated with TP ($P < 0.01$; Figure S3e) and Olsen P ($P < 0.05$; Figure S3f) and negatively correlated with

total C:P ($P < 0.01$; Figure S3g) and total N:P ($P < 0.05$; Figure S3h) ratios, under both forms of P type, across N treatments. Acid PME activity was correlated negatively with Olsen P concentration with addition of KH_2PO_4 and positively correlated in the $\text{Ca}(\text{H}_2\text{PO}_4)_2$ treatment ($P < 0.05$; Figure S3i). Alkaline PME activity was positively correlated with Olsen P concentration with addition of KH_2PO_4 ($P = 0.001$; Figure S3j).

Under addition of KH_2PO_4 , activities of BG, NAG and alkaline PME were positively associated with the first axis of the RDA, together with plant P uptake, plant biomass and TP, Olsen P and TIN concentrations; activity of acid PME was negatively associated, together with SOC concentration (Figure 5a). Activities of BG, NAG and acid PME under addition of $\text{Ca}(\text{H}_2\text{PO}_4)_2$ were correlated with plant P uptake and Olsen P and TP concentrations, whereas activity of alkaline PME was correlated with pH (Figure 5b). Overall variation in enzymatic activities under the addition of KH_2PO_4 tended to be driven by plant P uptake, plant biomass and concentrations of Olsen-P, TP, SOC and TIN that, together, explained 42.2% of the total variation (Fig. 5a). In contrast, soil pH, plant P uptake and Olsen-P and TP concentrations explained 25.5% of the variation in enzymatic activities under the addition of $\text{Ca}(\text{H}_2\text{PO}_4)_2$ (Figure 5b).

Discussion

Microbial biomass PMBP concentration increased under the two chemical forms of P, irrespective of addition of N, indicating immobilization of P in microbial biomass and limitation of P in this typical meadow steppe ecosystem. It is likely that alleviation of microbial P limitation would trigger the activity of extracellular enzymes, because our

multivariate analyses showed that P stocks and availability and plant P uptake were key drivers of the increases in enzymatic activities.

Effects of P on enzymatic activities

Our study is one of few that have reported increases in BG activity in response to greater availability of P, and thus far the only study in the cold to mid-temperate transitional climatic zone (Table 1). We detected a positive effect of P on BG activity, indicating soil-C cycling in this meadow steppe may be constrained by P availability; this finding supported our first hypothesis. However, the effect of P depended on its chemical form, because BG activity was greater with increasing rate of KH_2PO_4 but unaffected by $\text{Ca}(\text{H}_2\text{PO}_4)_2$. This finding was supported by the positive correlations of BG activity with soil TP and with Olsen P concentrations when KH_2PO_4 was added, and the lack of such associations under addition of $\text{Ca}(\text{H}_2\text{PO}_4)_2$ (Figure S3a and b). It is also possible that optimal BG activity decreased with the lower pH levels recorded under the addition of $\text{Ca}(\text{H}_2\text{PO}_4)_2$. The addition of the less soluble $\text{Ca}(\text{H}_2\text{PO}_4)_2$ may have reduced decomposition rates, because microbial BG activity was lower than in soils treated with the more soluble KH_2PO_4 .

Previous studies in wetland and alpine meadow soils found that BG activity was unaffected by P loading availability, but positively correlated with DOC concentration (Wright & Reddy, 2001; Jing *et al.*, 2016). In our study, litterfall biomass, but not DOC concentration, positively affected BG activity, regardless of form of P, indicating that plant litter played a more important role than DOC concentration in the regulation of

369 BG activity. ~~Higher-Increased~~ levels of soil N and P may ~~increase-improve~~ substrate
370 quality, such as ~~rlower-educed~~ litterfall C:N and C:P, and increase quantity (as litterfall
371 biomass) (Hobbie, 2005; Li *et al.*, 2017). The negative correlations between soil C:P
372 and N:P with BG activity (Figures S3c and d) support the premise that substrate quality
373 plays an important role in the regulation of enzymatic activities (Wallenstein *et al.*, 2009;
374 Phillips *et al.*, 2014). Indeed, litter contains abundant cellulose and hemicellulose that
375 then serve as substrates and induce BG activity (Allison *et al.*, 2013; Sinsabaugh *et al.*,
376 2008); however, dissolved organic matter (including DOC) is a enzymatic product of
377 litter decomposition that may inhibit BG activity (Tian *et al.*, 2010). We found that BG
378 activity was stimulated by the increased N and P inputs, likely due to the direct positive
379 roles of P and N availability in the synthesis of proteins and soil enzymes (Sinsabaugh
380 *et al.*, 2014; Tian *et al.*, 2016).

381 Addition of P led to an increase in microbial N demand, as indicated by the greater
382 levels of NAG activity (regardless of form of P), which support our first hypothesis.
383 Microbial NAG activity may eventually be subjected to soil C limitation in this meadow
384 steppe, because we found that increased application of the more soluble KH_2PO_4
385 decreased the concentration of SOC that was possibly linked to an increase in
386 decomposition. Indeed, addition of P also increased loss of soil C by increasing SOC
387 mineralization in Swedish meta-replicated long-term field experiments (Poeplau *et al.*,
388 2016a, b).

389 The positive effects of soil P (TP and Olsen P) levels on BG and NAG activities
390 contrasted with the lack of effects reported from a meta-analysis of 17 studies of tropical

ecosystems (Waring *et al.*, 2014). Paradoxically, microbial enzyme activities may be constrained by P in relatively fertile chernozems, but not in highly weathered and P-limited tropical soils, and it is possible these contrasting results may be due to differences in data synthesis from large-scale ecosystems and small-scale field-manipulative experiments (Niu *et al.*, 2016). Contrasting correlations between Olsen P concentration and acid PME activity under addition of KH_2PO_4 (negative) and $\text{Ca}(\text{H}_2\text{PO}_4)_2$ (positive) may have been due to the greater levels of plant P demand under $\text{Ca}(\text{H}_2\text{PO}_4)_2$ addition (Figure S2c) that are usually associated with low levels of Olsen P and high levels of PME activities (Antibus *et al.*, 1992). Soil P parameters have been reported to positively (Colvan *et al.*, 2001; Tian *et al.*, 2016) and negatively (Olander & Vitousek, 2000; Phoenix *et al.*, 2004) affect PME activity, where responses may depend on effects of initial levels of soil P, plant productivity, intensity of P uptake by plants, and soil properties on abiotic P fixation (Tian *et al.*, 2016; Margalef *et al.*, 2017). In our study, the increase in alkaline PME activity, even with exogenous P inputs, indicated that microbial P demand was stimulated with nutrient addition. We found a lack of response in aboveground biomass to addition of P (Figure S1b). Nevertheless, the increase in microbial P demand and uptake, as supported by the observed rise in MBP under fertilization with the two forms of P, could have diminished the ability of root biomass to successfully outcompete microbes for P (Marschner *et al.*, 2011). Inconsistent changes in MBC concentrations and enzymatic activities indicate a decoupling of the size and activity of the microbial community under elevated nutrient inputs (Lori *et al.*, 2017), and asymmetric changes in MBC with MBP concentrations

indicate that soil microorganisms may preferentially immobilize P (Bünemann *et al.*, 2012) and are stoichiometrically plastic (Xu *et al.*, 2013) in response to nutrient inputs.

Chemical form of P only affected alkaline PME activity, partially supporting our second hypothesis, where we found greater levels of alkaline PME activity in the $\text{Ca}(\text{H}_2\text{PO}_4)_2$ treatment that were associated with lower levels of Olsen P. The RDA indicated that drivers of enzymatic activity differed between the two forms of P (Figure 5) and the overall contrasting effects of P form were likely caused by differences in soil environments and soil-plant interactions, such as the rate and intensity of P uptake.

Effects of N on enzymatic activities

We found that BG activity increased with elevated N, indicating that greater availability of N alleviated microbial N limitation and stimulated microbial BG activity, thus supporting our first hypothesis. Although evidence that availability of N increases BG activity has been reported from other grassland and forest ecosystems (Henry *et al.*, 2005; Keeler *et al.*, 2009), our results contrast with those from semi-arid steppe grasslands in Inner Mongolia, where BG activity decreased with N addition (Wei *et al.*, 2013; Yang *et al.*, 2017). This discrepancy may be due to differences in effects of temperature, precipitation and soil fertility on the decomposition of plant residues and supply of C to microorganisms. For example, the meadow steppe is less water-limited than the semi-arid steppe, and an increase in soil moisture in Inner Mongolian grasslands has been reported to alleviate soil acidification, due to a reduction in leaching of basic cations (Cai *et al.*, 2017), and physiological stress in soil microorganisms

caused by atmospheric N deposition (Zhang *et al.*, 2015; Yang *et al.*, 2017). Thus, improved water conditions in wetter meadow steppes may interact with higher N availability to promote microbial growth and BG activity. Our finding that acid PME activity increased with elevated N indicated associated increases in P limitation and microbial and plant demand for P. Given that mineralization of C is the first step in P mineralization, where the hydrolysis of large C polymers facilitates the enzymatic catalysis of P-C and N-C hydrolysis, it is likely that increased BG activity may lead to subsequent P mineralization.

Although the increase in NAG activity with N addition was unexpected, positive effects of N on NAG activity have been detected in bulk soil (Yang *et al.*, 2017) and soil fractions (Wang *et al.*, 2015) in a semi-arid steppe ecosystem. The addition of N may have enhanced plant N uptake that increased plant productivity (Hodge *et al.*, 2000) and microbial N demand. According with this, increases in the rates of litter decomposition associated to soil enzyme activities (including N-cycle enzymes) has been observed in response to N-addition (Wang *et al.*, 2011). A recent meta-data analysis indicated how N fertilization increases the activities of hydrolase and oxidase enzymes, related to an increase in litter production due to higher plant production under higher levels of N-availability (Jian *et al.*, 2016). Increased NAG activity may derive from increases in mycorrhizal biomass for higher P transportation, possibly in response to higher plant P demand under elevated N (Miller *et al.*, 1998; Henry *et al.*, 2005). However, the increase in aboveground plant biomass (Figure S1b) coupled with a decrease in AMF biomass (Figure S2a) under the addition of N indicated more effective

competition by plants for N, resulting in N-limitation among the soil microorganisms, especially AMF, that then led to increased NAG production with greater plant density and productivity.

The divergent responses of acid and alkaline PME activities to N addition in this study may be due to greater levels of plant productivity (Figure S2b) and plant P uptake (Figure S2c) and indicate that PME production may have derived from different sources; for example, acid PME is produced by plant roots and soil microbes, whereas alkaline PME is principally produced by soil microbes (Tabatabai, 1994). Therefore, stable acid PME activity may be the consequence of a tradeoff between plant and soil microbial demand for P due to N enrichment. The greater levels of alkaline PME activity under N addition infer greater microbial P demand as a result of superior competition by plants for P (Marschner *et al.*, 2011), as supported by the greater levels of plant biomass and plant P uptake (Figures S2b and c) and unaffected MBC (Table S2) and MBP concentrations (Figure 1) in response to elevated N.

Stoichiometric traits of soil enzymes

The extracellular enzyme model (Moorhead *et al.*, 2012) and data collected from globally distributed soils and freshwater sediments (Sinsabaugh *et al.*, 2009) have demonstrated that the ratios of the activities of C-, N- and P-acquiring enzymes approximately converge to 1. Usually, soil microbial growth is more limited by C than N or P (Allison *et al.*, 2010); however, enzymatic activity is not always correlated with nutrient requirements for microbial growth, as indicated by our data. The SMA analysis

indicated that microbial activity in the grassland was more co-limited by N and P than the global average (Figure 3; Table S4). Indeed, it has been shown that N limitation constrains grassland productivity (Ren *et al.*, 2017) and microbial activity (Henry *et al.*, 2005), whereas P limitation of productivity may be gradual, as indicated by the globally decreasing soil P pool across grassland soils, due to intensified forage production and food supply (Sattari *et al.*, 2012, 2016). Thus, N and P fertilization may be necessary to maintain fertility in grassland soils (Sattari *et al.*, 2012), because increases in atmospheric N and P deposition may not be sufficient. Under this scenario, it is likely that greater levels of ~~large-scale~~large-scale ecosystem nutrient inputs would facilitate microbial activity that may then affect plant nutrition and soil C sequestration. The optimal amounts of P addition in this grassland ecosystem is suggested to be 6 g P m⁻² yr⁻¹ as shown by the relatively higher extracellular enzyme activities and potentially enhanced nutrient cycling rates at this P input level. Enzymatic stoichiometry may be a more reliable indicator of microbial nutrient limitation than microbial biomass C:N:P ratios (Xu *et al.*, 2013), due to the functional role of enzymes in the uptake and cycling of nutrients that sustain ecosystem productivity (Sinsabaugh & Shah, 2011).

Conclusions

Availability of N and P elicited positive effects on the activities of BG, NAG and alkaline PME; alkaline PME activity was lower under the more soluble KH₂PO₄. Elevated N input stimulated plant productivity and P uptake and led to soil microbial P limitation that was greater in the Ca(H₂PO₄)₂, as indicated by higher levels of alkaline

PME activity. Addition of N increased activities of BG, NAG and alkaline PME by increasing substrate availability, potentially increasing plant-microbe competition for C and N and intensity of plant P uptake. Our data indicated that KH_2PO_4 mediated changes in enzymatic activities tended to be highly and positively associated with soil P availability and intensity of plant P uptake, while $\text{Ca}(\text{H}_2\text{PO}_4)_2$ mediated changes in soil pH played a more essential role in enzymatic activities than plant P uptake. The activities of soil enzymes in the study grassland were principally determined by P availability and plant P content, indicating anthropogenic changes in ecosystem N and P levels may elicit similar effects on soil enzymes, but that will likely depend on the chemical form of P fertilizer.

Acknowledgements

This study was financially supported by the National Natural Science Foundation of China (31770525), the National Key Research and Development Program of China (2016YFC0500707, 2016YFC0500X01) and the European Research Council Synergy grant (ERC-SyG-2013-610028, IMBALANCE-P).

Data accessibility

Data sets may be obtained from the corresponding author.

References

Allison, S.D., Weintraub, M.N., Gartner, T.B., Waldrop, M.P., 2010. Evolutionary-

523 economic principles as regulators of soil enzyme production and ecosystem
 524 function. In: Shukla, G., Varma, A. (Eds). Soil enzymology. Springer-Verlag Berlin
 525 Heidelberg, pp. 229-243.

526 Allison, S.D., Lu, Y., Weihe, C., Goulden, M.L., Martiny, A.C., Treseder, K.K., Martiny,
 527 J.B., 2013. Microbial abundance and composition influence litter decomposition
 528 response to environmental change. *Ecology* 94, 714-725.

529 Alvarez-Clare, S., Mack, M.C., Brooks, M., 2013. A direct test of nitrogen and
 530 phosphorus limitation to net primary productivity in a lowland tropical wet
 531 forest. *Ecology* 94, 1540–1551.

532 Bai, Y., Wu, J., Clark, C. M., Naeem, S., Pan, Q., Huang, J., Zhang, L., Han, X., 2010.
 533 Tradeoffs and thresholds in the effects of nitrogen addition on biodiversity and
 534 ecosystem functioning: evidence from inner Mongolia Grasslands. *Global Change*
 535 *Biology* 16, 358-372.

536 Bilyera, N., Blagodatskaya, E., Yevdokimov, I., Kuzyakov, Y., 2018. Towards a
 537 conversion factor for soil microbial phosphorus. *European Journal of Soil Biology*
 538 87, 1-8.

539 Binkley, D., Högberg, P., 2016. Tamm review: revisiting the influence of nitrogen
 540 deposition on Swedish forests. *Forest Ecology and Management* 368, 222-239.

541 Brookes, P.C., Powlson, D.S., Jenkinson, D.S., 1982. Measurement of microbial
 542 biomass phosphorus in soil. *Soil Biology & Biochemistry* 14, 319-329.

543 Bünemann, E.K., Oberson, A., Liebisch, F., Keller, F., Annaheim, K.E., Huguenin-Elie,
 544 O., Frossard, E., 2012. Rapid microbial phosphorus immobilization dominates

gross phosphorus fluxes in a grassland soil with low inorganic phosphorus availability. *Soil Biology & Biochemistry* 51, 84-95.

Cai, J., Luo, W., Liu, H., Feng, X., Zhang, Y., Wang, R., Xu, Z., Zhang, Y., Jiang, Y., 2017. Precipitation-mediated responses of soil acid buffering capacity to long-term nitrogen addition in a semi-arid grassland. *Atmospheric Environment* 170, 312-318.

Carreiro, M.M., Sinsabaugh, R.L., Repert, D.A., Parkhurst, D.F., 2000. Microbial enzyme shifts explain litter decay responses to simulated nitrogen deposition. *Ecology* 81, 2359-2365.

Cenini, V.L., Fornara, D.A., McMullan, G., Ternan, N., Lajtha, K., Crawley, M.J., 2015. Chronic nitrogen fertilization and carbon sequestration in grassland soils: evidence of a microbial enzyme link. *Biogeochemistry* 126, 301-313.

Chen, J., Luo, Y., Li, J., Zhou, X., Cao, J., Wang, R. W., Wang, Y., Shelton, S., Jin, Z., Walker L. M., Feng, Z., Niu, S., Feng, W., Jian, S., Zhou, L., 2017. Costimulation of soil glycosidase activity and soil respiration by nitrogen addition. *Global Change Biology* 23, 1328-1333.

Colvan, S., Syers, J., O'Donnell, A., 2001. Effect of long-term fertiliser use on acid and alkaline phosphomonoesterase and phosphodiesterase activities in managed grassland. *Biology and Fertility of Soils* 34, 258-263.

Craine, J.M., Morrow, C., Stock, W.D., 2008. Nutrient concentration ratios and co-limitation in South African grasslands. *New Phytologist* 179, 829-836.

Craine, J.M., Jackson, R.D., 2010. Plant nitrogen and phosphorus limitation in 98 North

567 American grassland soils. *Plant and Soil* 334, 73-84.

568 Fernández-Martínez, M., Vicca, S., Janssens, I.A., Sardans, J., Luyssaert, S., Campioli,
569 M., Chapin III, F.S., Ciais, P., Malhi, Y., Obersteiner, M., Papale, D., Piao, S.,
570 Reichstein, M., Peñuelas, J., 2014. Nutrient availability as the key regulator of
571 global forest carbon balance. *Nature Climate Change* 4, 471-476.

572 Henry, H.A., Juarez, J.D., Field, C.B., Vitousek, P.M., 2005. Interactive effects of
573 elevated CO₂, N deposition and climate change on extracellular enzyme activity
574 and soil density fractionation in a California annual grassland. *Global Change*
575 *Biology* 11, 1808-1815.

576 Hobbie, S.E., 2005. Contrasting effects of substrate and fertilizer nitrogen on the early
577 stages of litter decomposition. *Ecosystems* 8, 644-656.

578 Hodge, A., Robinson, D., Fitter, A., 2000. Are microorganisms more effective than
579 plants at competing for nitrogen? *Trends in Plant Science* 5, 304-308.

580 IUSS Working Group WRB: World Reference Base for Soil Resources 2014.
581 International soil classification system for naming soils and creating legends for
582 soil maps. *World Soil Resources Reports No. 106*, FAO, Rome, 2014.

583 Jian, S., Li, J., Chen, J., Wang, G., Mayes, M. A., Dzantor, K.E., Hui, D., Luo, Y., 2016.
584 Soil extracellular enzyme activities, soil carbon and nitrogen storage under
585 nitrogen fertilization: A meta-analysis. *Soil Biology & Biochemistry* 101, 32-43.

586 Jing, X., Yang, X., Ren, F., Zhou, H., Zhu, B., He, J.S., 2016. Neutral effect of nitrogen
587 addition and negative effect of phosphorus addition on topsoil extracellular
588 enzymatic activities in an alpine grassland ecosystem. *Applied Soil Ecology* 107,

589 205-213.

590 Keeler, B.L., Hobbie, S.E., Kellogg, L.E., 2009. Effects of long-term nitrogen addition
591 on microbial enzyme activity in eight forested and grassland sites: implications for
592 litter and soil organic matter decomposition. *Ecosystems* 12, 1-15.

593 Liebisch, F., Keller, F., Huguenin-Elie, O., Frossard, E., Oberson, A., Bünemann, E. K.,
594 2014. Seasonal dynamics and turnover of microbial phosphorus in a permanent
595 grassland. *Biology and Fertility of Soils* 50, 465-475.

596 Li, H., Yang, S., Xu, Z., Yan, Q., Li, X., van Nostrand, J.D., He, Z., Yao, F., Han, X.,
597 Zhou, J., Deng, Y., Jiang, Y., 2017. Responses of soil microbial functional genes to
598 global changes are indirectly influenced by aboveground plant biomass
599 variation. *Soil Biology & Biochemistry* 104, 18-29.

600 Lori, M., Symnaczik, S., Mäder, P., De Deyn, G., Gattinger, A., 2017. Organic farming
601 enhances soil microbial abundance and activity-A meta-analysis and meta-
602 regression. *PLoS ONE* 12, e0180442.

603 Margalef, O., Sardans, J., Fernández-Martínez, M., Molowny-Horas, R., Janssens, I.A.,
604 Ciais, P., Goll, D., Richter, A., Obersteiner, M., Peñuelas, J., 2017. Global patterns
605 of phosphatase activity in natural soils. *Scientific Reports* 7, 1337.

606 Marschner, P., Crowley, D., Rengel, Z., 2011. Rhizosphere interactions between
607 microorganisms and plants govern iron and phosphorus acquisition along the root
608 axis—model and research methods. *Soil Biology & Biochemistry* 43, 883-894.

609 Mganga, K. Z., Razavi, B. S., Kuzyakov, Y., 2015. Microbial and enzymes response to
610 nutrient additions in soils of Mt. Kilimanjaro region depending on land

611 use. *European Journal of Soil Biology* 69, 33-40.

612 Marschner, P., Crowley, D., Rengel, Z., 2011. Rhizosphere interactions between
 613 microorganisms and plants govern iron and phosphorus acquisition along the root
 614 axis—model and research methods. *Soil Biology & Biochemistry* 43, 883-894.

615 Miller, M., Palojarvi, A., Rangger, A., Reeslev, M., Kjøller, A., 1998. The use of
 616 fluorogenic substrates to measure fungal presence and activity in soil. *Applied and
 617 Environmental Microbiology* 64, 613-617.

618 Moorhead, D.L., Lashermes, G., Sinsabaugh, R.L., 2012. A theoretical model of C-and
 619 N-acquiring exoenzyme activities, which balances microbial demands during
 620 decomposition. *Soil Biology & Biochemistry* 53, 133-141.

621 Mori, T., Lu, X., Aoyagi, R., Mo, J., 2018. Reconsidering the phosphorus limitation of
 622 soil microbial activity in tropical forests. *Functional Ecology* 32:1145-1154.

623 Murphy, J., Riley, J.P., 1962. A modified single solution method for the determination
 624 of phosphate in natural waters. *Analytica Chimica Acta* 27, 31-36.

625 Nannipieri, P., Trasar-Cepeda, C., Dick, R.P., 2018. Soil enzyme activity: a brief history
 626 and biochemistry as a basis for appropriate interpretations and meta-analysis.
 627 *Biology and Fertility of Soils* 54, 11-19.

628 Niklas, K.J., 2006. A phyletic perspective on the allometry of plant biomass-partitioning
 629 patterns and functionally equivalent organ-categories. *New Phytologist* 171, 27-40.

630 Niu, S., Classen, A. T., Dukes, J.S., Kardol, P., Liu, L., Luo, Y., Rustad, L., Sun, J., Tang,
 631 J., Templer, P.H., Thomas, R.Q., Tian, D., Vicca, S., Wang, Y., Xia, J., Zaehle, S.,
 632 2016. Global patterns and substrate-based mechanisms of the terrestrial nitrogen

633 cycle. *Ecology Letters* 19, 697-709.

634 Olander, L.P., Vitousek, P.M., 2000. Regulation of soil phosphatase and chitinase
635 activity by N and P availability. *Biogeochemistry* 49, 175-190.

636 Parham, J.A., Deng, S.P., 2000. Detection, quantification and characterization of β -
637 glucosaminidase activity in soil. *Soil Biology & Biochemistry* 32, 1183-1190.

638 Peñuelas, J., Sardans, J., Rivas-ubach, A., Janssens, I.A., 2012. The human-induced
639 imbalance between C, N and P in Earth's life system. *Global Change Biology* 18,
640 3-6.

641 Peñuelas, J., Poulter, B., Sardans, J., Ciais, P., van der Velde, M., Bopp, L., Boucher,
642 O., Godderis, Y., Hinsinger, P., Llusia, J., Nardin, E., Vicca, S., Obersteiner, M.,
643 Janssens, I.A., 2013. Human-induced nitrogen-phosphorus imbalances alter natural
644 and managed ecosystems across the globe. *Nature Communications* 4, 2934.

645 Phillips, L.A., Ward, V., Jones, M.D., 2014. Ectomycorrhizal fungi contribute to soil
646 organic matter cycling in sub-boreal forests. *The ISME Journal* 8, 699.

647 Phoenix, G.K., Booth, R.E., Leake, J.R., Read, D.J., Grime, J.P., Lee, J.A., 2003. Effects
648 of enhanced nitrogen deposition and phosphorus limitation on nitrogen budgets of
649 semi - natural grasslands. *Global Change Biology* 9, 1309-1321.

650 Poeplau, C., Bolinder, M.A., Kirchmann, H., Kätterer, T., 2016a. Phosphorus
651 fertilisation under nitrogen limitation can deplete soil carbon stocks: evidence from
652 Swedish meta-replicated long-term field experiments. *Biogeosciences* 13, 1119-
653 1127.

654 Poeplau, C., Herrmann, A.M., Kätterer, T., 2016b. Opposing effects of nitrogen and

655 phosphorus on soil microbial metabolism and the implications for soil carbon
656 storage. *Soil Biology & Biochemistry* 100, 83-91.

657 Ren, H., Xu, Z., Isbell, F., Huang, J., Han, X., Wan, S., Chen, S., Wang, R., Zeng, D.,
658 Jiang, Y., Fang, Y., 2017. Exacerbated nitrogen limitation ends transient stimulation
659 of grassland productivity by increased precipitation. *Ecological Monographs* 87,
660 457-469.

661 Sardans, J., Peñuelas, J., Estiarte, M., 2007. Seasonal patterns of root-surface
662 phosphatase activities in a Mediterranean shrubland. Responses to experimental
663 warming and drought. *Biology and Fertility of Soils* 43, 779-786.

664 Sardans, J., Alonso, R., Janssens, I. A., Carnicer, J., Vereseglou, S., Rillig, M. C.,
665 Fernández-Martínez, M., Sanders, T.G.M., Peñuelas, J., 2016. Foliar and soil
666 concentrations and stoichiometry of nitrogen and phosphorous across European
667 *Pinus sylvestris* forests: relationships with climate, N deposition and tree
668 growth. *Functional Ecology* 30, 676-689.

669 Schimel, J.P., Weintraub, M.N., 2003. The implications of exoenzyme activity on
670 microbial carbon and nitrogen limitation in soil: a theoretical model. *Soil Biology
671 & Biochemistry* 35, 549-563.

672 Shukla, G., Varma, A., 2011. *Soil enzymology*. Springer-Verlag, Berlin, Heidelberg.

673 Sinsabaugh, R. L., Lauber, C. L., Weintraub, M. N., Ahmed, B., Allison, S. D.,
674 Crenshaw, C., Contosta, A.R., Cusack, D., Frey, S., Gallo, M.E., Gartner, T.B.,
675 Hobbie, S.E., Holland, K., Keeler, B.L., Powers, J.S., Stursova, M., Takacs-
676 Vesbach, C., Waldrop, M.P., Wallenstein, M.D., Zak, D.R., Zeglin, L.H., 2008.

677 Stoichiometry of soil enzyme activity at global scale. *Ecology Letters* 11, 1252-
678 1264.

679 Sinsabaugh, R.L., Hill, B.H., Shah, J. J. F., 2009. Ecoenzymatic stoichiometry of
680 microbial organic nutrient acquisition in soil and sediment. *Nature* 462, 795-798.

681 Sinsabaugh, R.L., Shah, J.J.F., 2011. Ecoenzymatic stoichiometry of recalcitrant
682 organic matter decomposition: the growth rate hypothesis in
683 reverse. *Biogeochemistry* 102, 31-43.

684 Sinsabaugh, R.L., Belnap, J., Findlay, S.G., Shah, J.J.F., Hill, B.H., Kuehn, K.A., Kuske,
685 C.R., Litvak, M.E., Martinez, N.G., Moorhead, D.L., Warnock, D.D., 2014.
686 Extracellular enzyme kinetics scale with resource
687 availability. *Biogeochemistry* 121, 287-304.

688 Sattari, S.Z., Bouwman, A.F., Giller, K.E., van Ittersum, M.K., 2012. Residual soil
689 phosphorus as the missing piece in the global phosphorus crisis
690 puzzle. *Proceedings of the National Academy of Sciences* 109, 6348-6353.

691 Sattari, S.Z., Bouwman, A.F., Rodríguez, R.M., Beusen, A.H.W., Van Ittersum, M.K.,
692 2016. Negative global phosphorus budgets challenge sustainable intensification of
693 grasslands. *Nature Communications* 7, 10696.

694 Sterkenburg, E., Clemmensen, K.E., Ekblad, A., Finlay, R.D., Lindahl, B.D., 2018.
695 Contrasting effects of ectomycorrhizal fungi on early and late stage decomposition
696 in a boreal forest. *The ISME Journal* 12, 2187-2197.

697 Tabatabai, M., 1994. Soil enzymes. In: Bottomley, P.S., Angle, J.S., Weaver, R.W.
698 (Eds.), *Methods of soil analysis: Part 2-microbiological and biochemical properties*,

699 4th edn. Soil Science Society of America, Madison, pp 775-833.

700 Tian, L., Dell, E., Shi, W., 2010. Chemical composition of dissolved organic matter in
701 agroecosystems: correlations with soil enzyme activity and carbon and nitrogen
702 mineralization. *Applied Soil Ecology* 46, 426-435.

703 Tian, J., Wei, K., Condrón, L. M., Chen, Z., Xu, Z., Chen, L., 2016. Impact of land use
704 and nutrient addition on phosphatase activities and their relationships with organic
705 phosphorus turnover in semi-arid grassland soils. *Biology and Fertility of Soils* 52,
706 675-683.

707 Trivedi, P., Delgado-Baquerizo, M., Trivedi, C., Hu, H., Anderson, I.C., Jeffries, T. C.,
708 Zhou, J., Singh, B.K., 2016. Microbial regulation of the soil carbon cycle: evidence
709 from gene–enzyme relationships. *The ISME Journal* 10, 2593-2604.

710 Turner, B.L., Wright, S.J., 2014. The response of microbial biomass and hydrolytic
711 enzymes to a decade of nitrogen, phosphorus, and potassium addition in a lowland
712 tropical rain forest. *Biogeochemistry* 117, 115-130.

713 Vance, E.D., Brooks, P.C., Jenkinson, D.S., 1987. An extraction method for measuring
714 soil microbial biomass-C. *Soil Biology & Biochemistry* 19, 703-707.

715 Wallenstein, M.D., McMahon, S.K., Schimel, J.P., 2009. Seasonal variation in enzyme
716 activities and temperature sensitivities in Arctic tundra soils. *Global Change*
717 *Biology* 15, 1631-1639.

718 Wallenstein, M.D., Weintraub, M.N., 2008. Emerging tools for measuring and modeling
719 the in situ activity of soil extracellular enzymes. *Soil Biology & Biochemistry* 40,
720 2098-2106.

721 Wang, C., Han, G., Jia, Y., Feng, X., Guo, P., Tian, X., 2011. Response of litter
 722 decomposition and related soil enzyme activities to different forms of nitrogen
 723 fertilization in a subtropical forest. *Ecological Research* 26, 505-513.

724 Wang, D., Ba, L., 2008. Ecology of meadow steppe in northeast China. *The Rangeland*
 725 *Journal* 30, 247-254.

726 Wang, R., Filley, T. R., Xu, Z., Wang, X., Li, M. H., Zhang, Y., Luo, W., Jiang, Y., 2014.
 727 Coupled response of soil carbon and nitrogen pools and enzyme activities to
 728 nitrogen and water addition in a semi-arid grassland of Inner Mongolia. *Plant and*
 729 *Soil* 381, 323-336.

730 Wang, R., Dorodnikov, M., Yang, S., Zhang, Y., Filley, T. R., Turco, R. F., Zhang, Y.,
 731 Xu, Z., Li, H., Jiang, Y., 2015. Responses of enzymatic activities within soil
 732 aggregates to 9-year nitrogen and water addition in a semi-arid grassland. *Soil*
 733 *Biology & Biochemistry* 81, 159-167.

734 Wang, Y., Ciais, P., Goll, D., Huang, Y., Luo, Y., Wang, Y.P., Bloom, A.A., Broquet, G.,
 735 Hartmann, J., Peng, S., Peñuelas, J., Piao, S., Sardans, J., Stocker, B.D., Wang, R.,
 736 Zaehle, S., Zechmeister-Boltenstern, S., 2018. GOLUM-CNP v1.0: a data-driven
 737 modeling of carbon, nitrogen and phosphorus cycles in major terrestrial
 738 biomes. *Geoscientific Model Development* 11, 3903-3928.

739 Waring, B.G., Weintraub, S.R., Sinsabaugh, R.L., 2014. Eoenzymatic stoichiometry
 740 of microbial nutrient acquisition in tropical soils. *Biogeochemistry* 117, 101-113.

741 Wei, C., Yu, Q., Bai, E., Lü, X., Li, Q., Xia, J., Kardol, P., Liang, W., Wang, Z., Han,
 742 X., 2013. Nitrogen deposition weakens plant–microbe interactions in grassland

ecosystems. *Global Change Biology* 19, 3688-3697.

Wright, A.L., Reddy, K.R., 2001. Phosphorus loading effects on extracellular enzyme activity in Everglades wetland soils. *Soil Science Society of America Journal* 65, 588–595.

Xu, X., Thornton, P.E., Post, W.M., 2013. A global analysis of soil microbial biomass carbon, nitrogen and phosphorus in terrestrial ecosystems. *Global Ecology and Biogeography* 22, 737-749.

Xu, W., Luo, X.S., Pan, Y., Zhang, L., Tang, A. H., Shen, J.L., Zhang, Y., Li, K., Wu, Q., Yang, D.W., Zhang, Y.Y., Xue, J., Li, W.Q., Li, Q.Q., Tang, L., Lu, S.H., Liang, T., Tong, Y.A., Liu, P., Zhang, Q., Xiong, Z.Q., Shi, X.J., Wu, L.H., Shi, W.Q., Tian, K., Zhong, X.H., Shi, K., Tang, Q.Y., Zhang, L.J., Huang, J.L., He, C.E., Kuang, F.H., Zhu, B., Liu, H., Jin, X., Xin, Y.J., Shi, X.K., Du, E.Z., Dore, A.J., Tang, S., Collett Jr., J.L., Goulding, K., Sun, Y.X., Ren, J., Zhang, F.S., Liu, X. (2015). Quantifying atmospheric nitrogen deposition through a nationwide monitoring network across China. *Atmospheric Chemistry and Physics* 15, 12345-12360.

Yang, S., Xu, Z., Wang, R., Zhang, Y., Yao, F., Zhang, Y., Turco, R.F., Jiang, Y., Zou, H., Li, H., 2017. Variations in soil microbial community composition and enzymatic activities in response to increased N deposition and precipitation in Inner Mongolian grassland. *Applied Soil Ecology* 119, 275-285.

Zhang, N., Wan, S., Guo, J., Han, G., Gutknecht, J., Schmid, B., Yu, L., Liu, W., Bi, Y., Wang, W., Ma, K., 2015. Precipitation modifies the effects of warming and nitrogen addition on soil microbial communities in northern Chinese grasslands. *Soil*

765 Biology & Biochemistry 89, 12-23.

766

Table 1 Literature review of effects of phosphorus (P) addition on soil β -glucosidase (BG), *N*-acetyl-glucosaminidase (NAG) and acid and alkaline phosphomonoesterases (PMEs) activity in grassland ecosystems.

Grassland type and location	P form	BG	NAG	Acid PME	Alkaline PME	Reference
Meadow grassland, UK	Triple superphosphate	–	–	0	↑	Colvan <i>et al.</i> 2001
Mesic grassland, Switzerland	Superphosphate	–	–	↓	–	Bünemann <i>et al.</i> 2012
Mesic grassland, Switzerland	Superphosphate	–	–	↓	–	Liebisch <i>et al.</i> 2014
Savannah, Tanzania	KH ₂ PO ₄	↑	↑	↑	–	Mganga <i>et al.</i> 2015
Semi-natural grassland, Tanzania	KH ₂ PO ₄	↑	↑	↑	–	
Alpine grassland, China	Triple superphosphate	0	0	0	–	Jing <i>et al.</i> 2016
Semi-arid steppe, China	Superphosphate	–	–	↑	↑	Tian <i>et al.</i> 2016
Old field, China	Superphosphate	–	–	↓	↓	

Effects annotated as ↑, ↓, 0 or – indicate positive, negative, no significant change or lack of data, respectively.

Figure captions

Figure 1 Effect of addition of KH_2PO_4 (a) or $\text{Ca}(\text{H}_2\text{PO}_4)_2$ (b) with 0 and 10 g N m⁻² y⁻¹ nitrogen (N) on concentration of microbial biomass phosphorus (MBP) (mean \pm SE, n = 5). Upper- and lowercase letters indicate differences among KH_2PO_4 and $\text{Ca}(\text{H}_2\text{PO}_4)_2$ treatments with and without added N, respectively.

Figure 2 Boxplots of activity of BG (a, b), NAG (c, d), acid PME (e, f) and alkaline PME (g, h) with addition of KH_2PO_4 (a, c, e, g) and $\text{Ca}(\text{H}_2\text{PO}_4)_2$ (b, d, f, h) with 0 (blue) and 10 g N m⁻² y⁻¹ (red). Different letters indicate differences among KH_2PO_4 and $\text{Ca}(\text{H}_2\text{PO}_4)_2$ treatments with or without added N, and asterisks indicate differences between N treatments for the rates of KH_2PO_4 and $\text{Ca}(\text{H}_2\text{PO}_4)_2$. Error bars indicate the 10th and 90th percentiles; black lines within the boxes represent median activity and the box limits indicate activity within the 25-75th percentile range.

Figure 3 Regression analyses of activities of NAG and BG, PME and BG and PME and NAG. All data are Ln-transformed. Dashed line: line of unity.

Figure 4 Mean litter biomass (\pm SE, n = 5) with addition of KH_2PO_4 (a) or $\text{Ca}(\text{H}_2\text{PO}_4)_2$ (b) under ambient and added nitrogen (N). Relationship between BG activity and litter biomass with addition of KH_2PO_4 (c) or $\text{Ca}(\text{H}_2\text{PO}_4)_2$ across N treatments (d). Upper- and lowercase letters indicate differences among KH_2PO_4 and $\text{Ca}(\text{H}_2\text{PO}_4)_2$ treatments with and without N addition, respectively. Asterisk indicates within P rate and type

differences between N treatments.

Figure 5 Redundancy analysis of the relationship between soil enzyme activity (BG, NAG, acid PME and alkaline PME) and explanatory parameters (plant P uptake, pH, plant biomass and TP, TIN, SOC and Olsen-P concentrations) (left) and their contributions to the variation in overall activity (right) under addition of KH_2PO_4 (a) or $\text{Ca}(\text{H}_2\text{PO}_4)_2$ (b) addition.

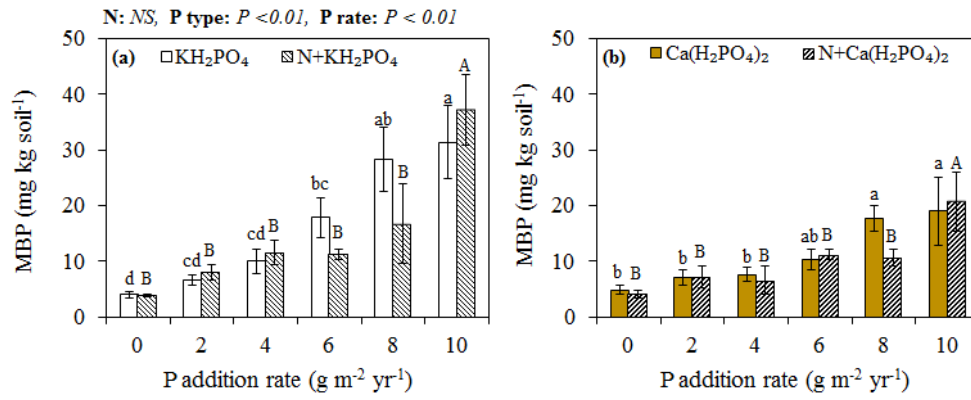


Figure 1

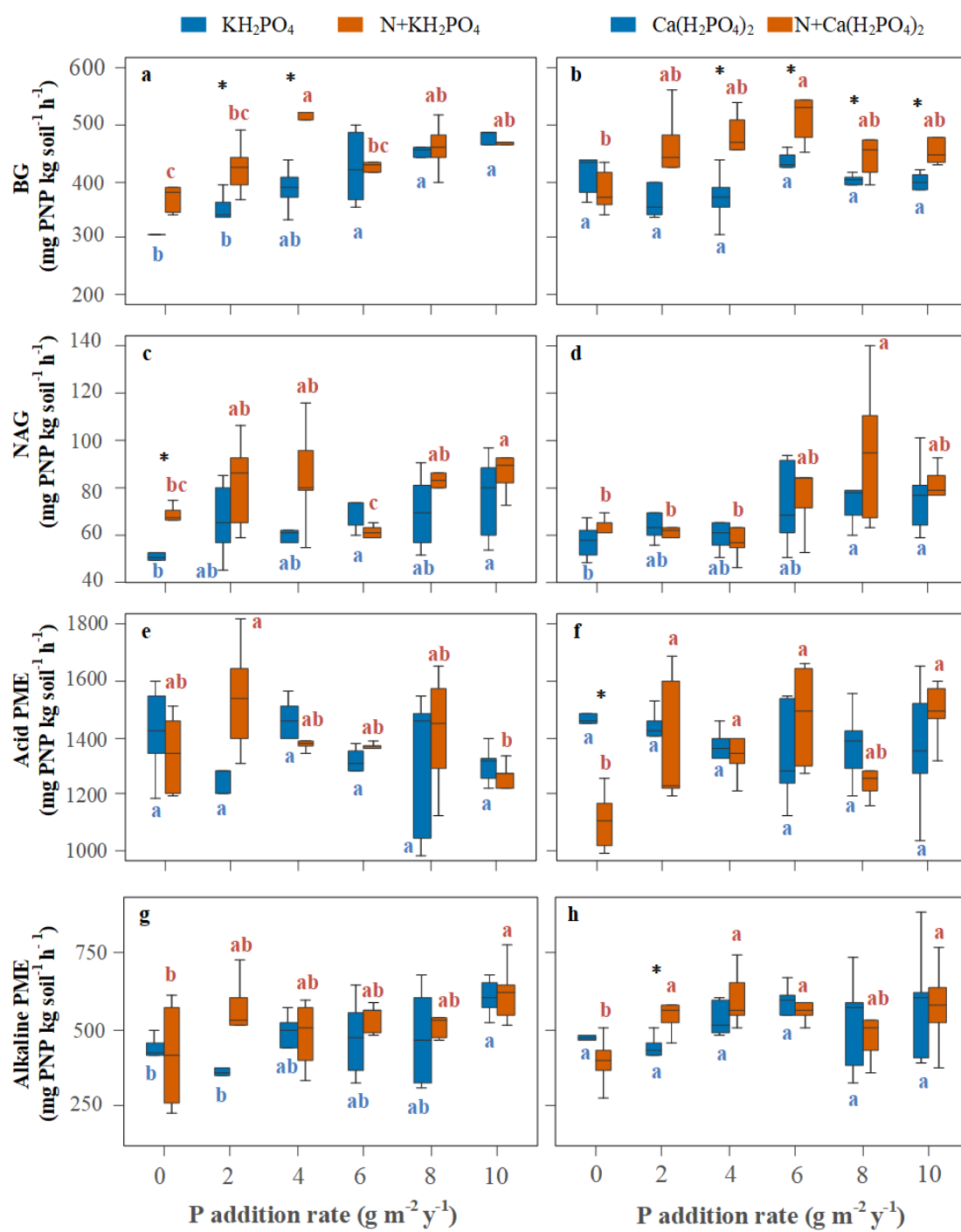


Figure 2

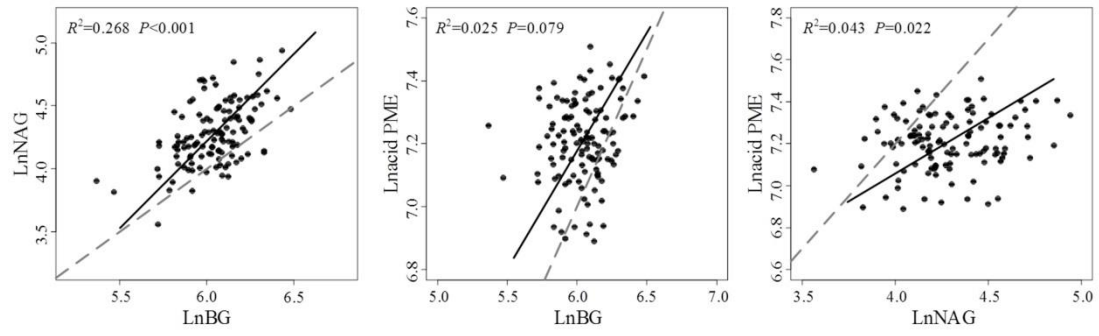


Figure 3

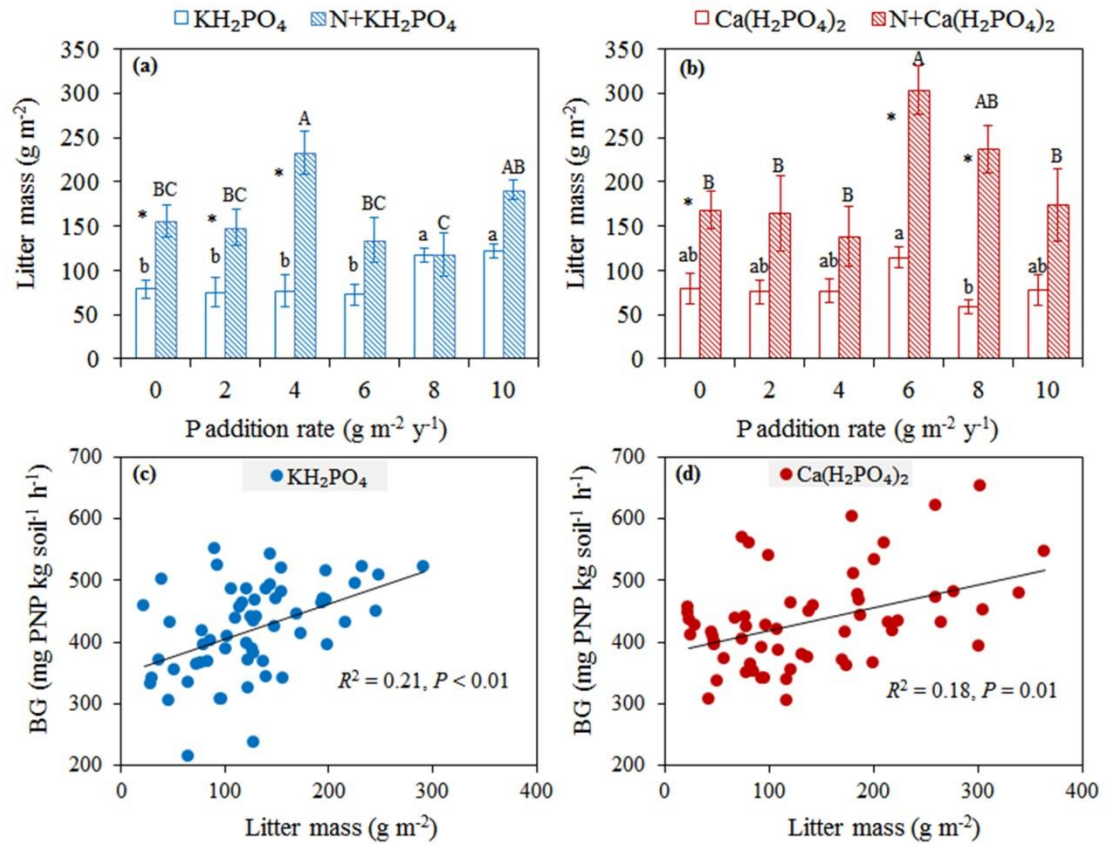


Figure 4

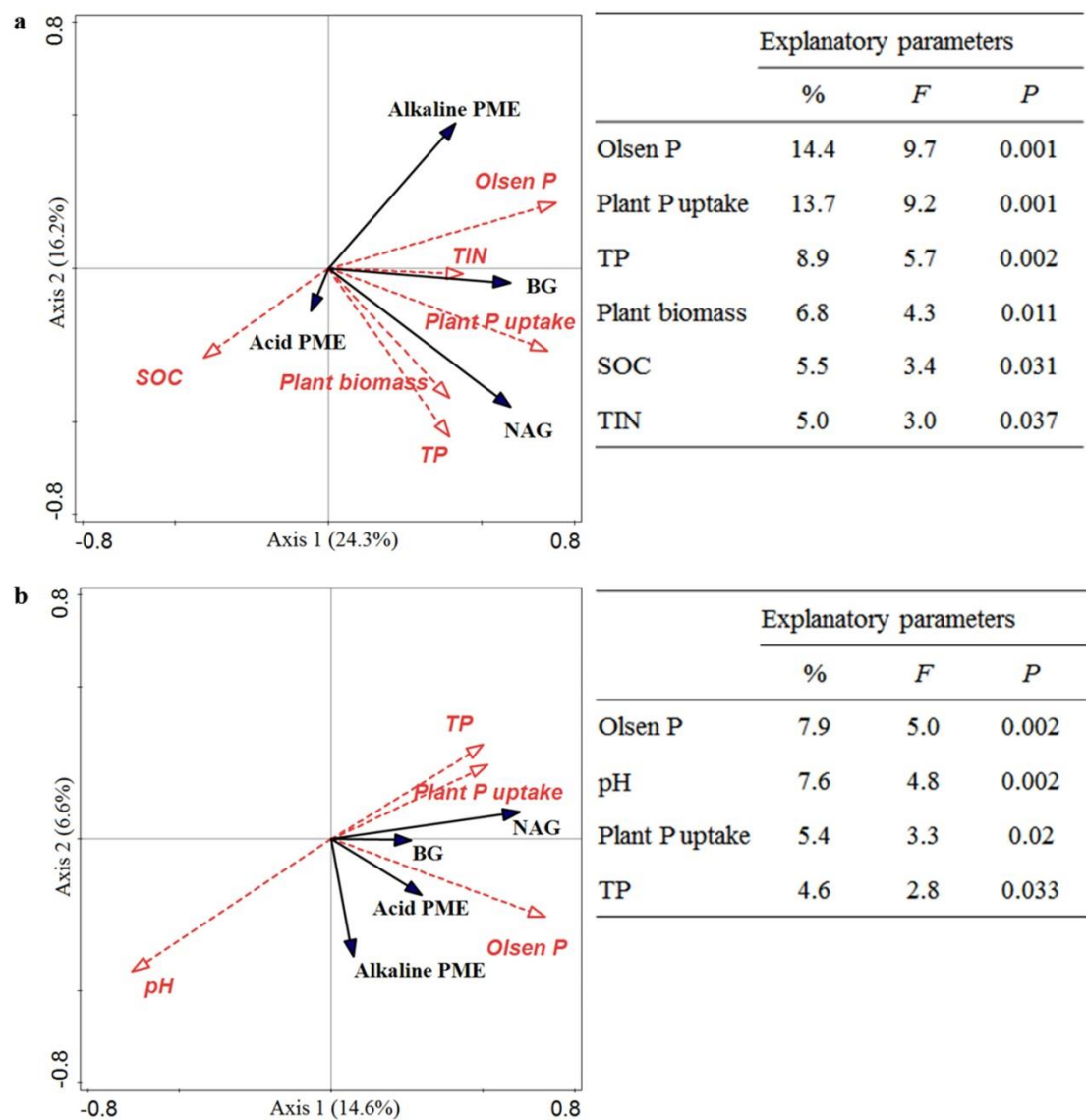


Figure 5